

Eosinophil-Selective Binding and Proapoptotic Effect in Vitro of a Synthetic Siglec-8 Ligand, Polymeric 6'-Sulfated Sialyl Lewis X

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ABSTRACT

The lectin Siglec-8 (sialic acid-binding, immunoglobulin-like lectin), which is selectively expressed on eosinophil surfaces and regulates eosinophil survival, preferentially binds to the glycan 6'-sulfo-sialyl Lewis X (6'-sulfo-sLe^x). Antibody engagement of Siglec-8 on eosinophils causes their apoptosis, suggesting that engagement of Siglec-8 with its natural glycan ligands in vivo may control allergic inflammation. We report that a soluble synthetic polymer displaying 6'-sulfo-sLe^x glycan selectively binds to human eosinophils and human embryonic kidney 293 cells expressing Siglec-8. Binding was inhibited by

anti-Siglec-8 antibody. In whole blood, eosinophils were the only leukocyte subtype to detectably bind polymeric 6'-sulfo-sLe^x. Interleukin-5-primed eosinophils underwent apoptosis when incubated with either anti-Siglec-8 monoclonal antibody or polymeric 6'-sulfo-sLe^x, although the glycan polymer was less effective. These data demonstrate that a soluble, multi-valent glycan selectively binds to human eosinophils and induces their apoptosis in vitro and provide proof-of-concept that such a reagent could be used to selectively target eosinophils.

Siglecs are a family of single-pass cell surface receptors that contain N-terminal sialic acid-binding domains (Varki and Angata, 2006; Crocker et al., 2007; von Gunten and Bochner, 2008). Among all of the known human Siglecs, each has a unique pattern of cell surface expression ranging from a single cell type, such as sialoadhesin (Siglec-1) on macrophages, to a wide range of cell types, such as CD33 (Siglec-3) on most leukocytes (Varki and Angata, 2006; Crocker et al.,

2007; von Gunten and Bochner, 2008). Somewhat more selective in cellular expression pattern is Siglec-8, found on eosinophils and mast cells and, to a lesser degree, on human basophils (Floyd et al., 2000; Kikly et al., 2000). Although all Siglecs bind sialic acid, each exhibits a distinct preference for the type of sialic acid, its glycosidic linkage, and the presence of underlying glycans (Varki and Angata, 2006; Crocker et al., 2007). However, whereas Siglec-8 binds weakly to 2,3-linked sialic acids (Floyd et al., 2000; Bochner et al., 2005), it shows a remarkable preference for 6'-sulfated-sialyl-Lewis X [6'-sulfo-sLe^x; NeuAc α 2-3Gal(6-O-SO₃) β 1-4(Fuc α 1-3)GlcNAc] without recognizing closely related glycans like sLe^x and 6-sulfo-sLe^x (Bochner et al., 2005). Because Siglec-8 engagement with specific antibodies induces eosinophil apoptosis (Nutku et al., 2003, 2005; Nutku-Bilir et al., 2008), it remains unknown whether a glycan ligand-based material would have a similar effect. Therefore, we determined whether a synthetic polymer-based ligand decorated with the 6'-sulfo-sLe^x glycan ligand of Siglec-8 would have the same cellular binding specificity and biology as that seen with monoclonal antibodies.

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Drs. Bochner and Schnaar are coauthors on existing and pending Siglec-8-related patents. If Siglec-8-related products are developed in the future, under a licensing agreement between GlaxoSmithKline and the Johns Hopkins University, Drs. Bochner and Schnaar may be entitled to a share of royalties received by the University on the potential sales of such products. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

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ABBREVIATIONS: sLe^x, sialyl Lewis X; HEK, human embryonic kidney; MFI, mean fluorescence intensity; PAA, polyacrylamide.

Materials and Methods

Generation of Stable Siglec-8-Transfected Human Embryonic Kidney 293 Cells. Human embryonic kidney 293 (HEK 293) cell stable transfectants expressing levels of Siglec-8 similar to levels found on eosinophils were prepared and maintained as described previously (Kikly et al., 2000; Yokoi et al., 2008).

Purification of Human Eosinophils. Human eosinophils were isolated from peripheral blood by density gradient centrifugation, red cell hypotonic lysis, and immunomagnetic negative selection as described previously (Matsumoto et al., 1995).

Polyacrylamide Conjugates. Thirty-kilodalton label-free and biotinylated conjugates were obtained as described previously (Bovin et al., 1993) by coupling aminopropyl glycosides to poly(4-nitrophenyl acrylate) followed by quenching of the polymer active groups with ethanolamine. The 2000-kDa polymers were obtained similarly starting from poly(4-*N*-hydroxysuccinimidyl acrylate) (Shilova et al., 2005). Spacer-armed tetrasaccharides 6'-sulfo-sLe^x, sLe^x, and 3'-sulfo-sLe^x were synthesized as described previously (Zemlyanukhina et al., 1995; Pazy-nina et al., 2003; G. V. Pazy-nina, V. Severov, M. L. Maisel, I. M. Belyanchikov, and N. V. Bovin, submitted for publication).

Flow Cytometry. Binding of Siglec-8 monoclonal antibodies 2E2, 2C4, and 9G4 (all murine IgG1, generously provided by Dr. John White, GlaxoSmithKline, Uxbridge, Middlesex, UK), sLe^x IgM monoclonal antibody FH6 (Bochner et al., 1994), a polyclonal sheep anti-human Siglec-8 antibody (Tateno et al., 2005), and appropriate isotype controls (all used at 10 µg/ml concentrations unless otherwise indicated) was determined in heparinized whole blood or on purified eosinophils by use of indirect immunofluorescence and flow cytometry as described previously (Kikly et al., 2000; Nutku et al., 2003). Binding of saturating concentrations (10 µg/ml) of biotinylated 30- or 2000-kDa 6'-sulfo-sLe^x-containing polyacrylamide (PAA) polymers and controls [30-kDa 3'-sulfo-sLe^x-PAA and sLe^x-PAA; 2000-kDa Lac-Nac-PAA (Rapoport et al., 2006; Tateno et al., 2007)] was detected by use of saturating concentrations of fluorescein isothiocyanate-streptavidin (R&D Systems, Minneapolis, MN) and flow cytometry. In some experiments, cells were preincubated as described previously (Nimrichter et al., 2008) for 90 min at 37°C with sialidase [either from *Clostridium perfringens*, 1.6 U/ml from Sigma-Aldrich (St. Louis, MO), or from *Vibrio cholerae*, 10 mU/ml from Roche Diagnostics (Indianapolis, IN)] or monoclonal or polyclonal Siglec-8 antibodies (20 min, 4°C) before incubation with various PAA materials.

Adhesion Assays. Biotinylated glycan-conjugated PAA polymers (at an optimal concentration of 10 µg/ml) were immobilized on streptavidin-coated 96-well plates (Pierce Chemical, Rockford, IL), and adhesion of calcein-AM-labeled HEK 293 cell suspensions was tested under static conditions (30 min, room temperature) essentially as described previously for eosinophils (Matsumoto et al., 1997). In some experiments, cells were preincubated with either monoclonal or polyclonal Siglec-8 antibodies before testing cell adhesion as indicated.

Apoptosis Assays. Purified eosinophils were cultured for 24 or 72 h in interleukin 5 (IL-5) (10 ng/ml, R&D Systems), and then either 10 µg/ml 2C4 antibody or 10 µg/ml 2000-kDa PAA polymers were added for an additional 24 h. None of the cultures was performed without IL-5 because cytokine exposure is needed to see apoptosis to Siglec-8 antibodies alone (Nutku et al., 2003; von Gunten et al., 2007; Nutku-Bilir et al., 2008). Thus, no secondary antibodies were included. Cell survival was then assessed by flow cytometric analysis after labeling with annexin-V and propidium iodide as described previously (Nutku et al., 2003; Nutku-Bilir et al., 2008).

Statistical Analyses. Paired two-tailed Student's *t* test was used to compare values, and results were considered significant for *p* values <0.05.

Results

6'-Sulfo-sLe^x-PAA Polymer Selectively Binds to Siglec-8.

Initial experiments used wild-type HEK 293 cells

and HEK 293 cells stably transfected with Siglec-8. As shown in Fig. 1, such transfected cells prominently and selectively label with Siglec-8 antibody (2E2) and also bind 30-kDa 6'-sulfo-sLe^x-PAA but not the closely related control glycan-PAA polymers (sLe^x and 3-sulfo-sLe^x) that differ from 6'-sulfo-sLe^x only by the absence of the sulfate residue or the sulfate attachment site. Further evidence of specificity of binding is shown in Fig. 2, where a polyclonal sheep Siglec-8 antibody blocked 6'-sulfo-sLe^x-PAA binding to the Siglec-8-HEK 293 cell transfectants. This differs from results obtained by use of a mixture of all three Siglec-8 monoclonal antibodies, which failed to inhibit polymer binding, demonstrating that the polyclonal reagent, unlike the monoclonal antibodies, recognizes the carbohydrate binding site. Consistent with this conclusion is the observation that preincubation of Siglec-8-expressing HEK 293 cells with polyclonal antibodies does not reduce binding of any of the monoclonal antibodies, or vice versa (data not shown).

Further evidence of the functionality of 6'-sulfo-sLe^x-PAA binding to Siglec-8 is shown in Fig. 3 by use of an adhesion assay in which this or various control 30-kDa polymers were immobilized on plastic surfaces, and adhesion of Siglec-8-transfected or mock-transfected HEK 293 cells was tested under static conditions. Significant attachment was only observed with HEK 293 cells transfected with Siglec-8 and immobilized 30-kDa 6'-sulfo-sLe^x-PAA polymer (*p* < 0.007), and adhesion was eliminated by preincubation of the HEK 293 transfectants with polyclonal Siglec-8 antibody but not any of the monoclonals (Fig. 3; data not shown).

Surprisingly, and in marked contrast, when this same 6'-sulfo-sLe^x-PAA 30-kDa polymer was tested for its ability to bind purified eosinophils, either in flow cytometry or in static adhesion assays, no detectable binding was seen, even though these cells expressed levels of Siglec-8 virtually identical to the HEK 293 transfectants, as determined by antibody labeling and flow cytometry (data not shown). Because previous studies suggest that endogenous cell surface sialic acids can bind in so-called "cis" configuration and block Siglec ligand binding (Crocker et al., 2007), eosinophils were pre-treated with sialidase to remove surface sialic acids (which was confirmed by complete loss of sLe^x surface expression by eosinophils), yet neither treated nor untreated eosinophils bound to the 30-kDa 6'-sulfo-sLe^x-PAA polymer (data not

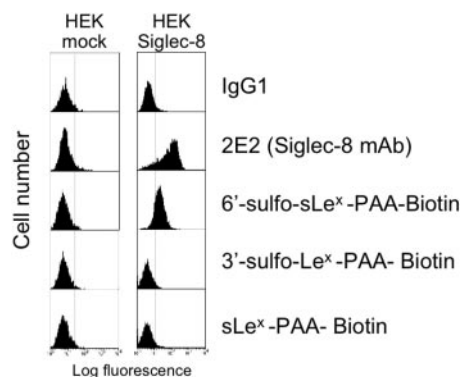


Fig. 1. HEK 293 cells stably transfected with Siglec-8 have the ability to bind 30-kDa 6'-sulfo-sLe^x-PAA polymer. Shown are flow cytometric histograms comparing mock-transfected HEK 293 cells that fail to bind Siglec-8 antibody and 6'-sulfo-sLe^x-PAA polymer. Also shown is the failure of binding of several structurally similar glycan-conjugated polymers. Results are representative of two experiments with similar results.

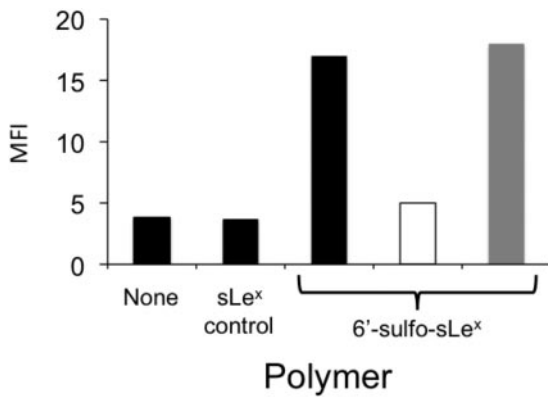


Fig. 2. Effect of different Siglec-8 antibodies on 30-kDa 6'-sulfo-sLe^x-PAA polymer binding to HEK 293 cells stably transfected with Siglec-8. Black bars represent data with no added antibodies. A polyclonal sheep Siglec-8 antibody had blocking activity (open bar), whereas a combination of mouse monoclonal antibodies (50 µg/ml each of 2E2, 2C4, and 9G4), had no blocking activity (gray bar). Results are from a single experiment representative of three experiments with similar results. MFI, mean fluorescence intensity.

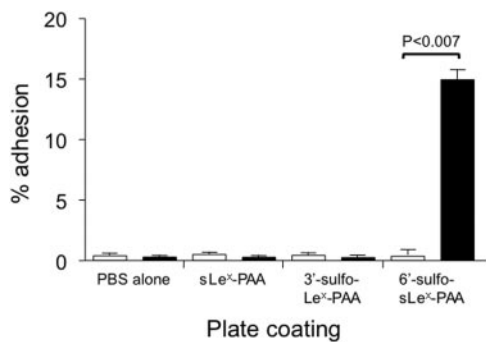


Fig. 3. Adhesion of mock-transfected HEK 293 cells (open bars) and HEK 293 cells stably transfected with Siglec-8 (black bars) to various immobilized substrates. Attachment was only observed when using 30-kDa 6'-sulfo-sLe^x-PAA polymer and HEK 293 cells transfected with Siglec-8. Data are means ± S.E.M. of triplicate determinations and are from *n* = 2–4 experiments.

shown). However, when a much larger (2000-kDa) 6'-sulfo-sLe^x-PAA polymer was tested, binding to purified eosinophils was readily detected (net mean fluorescence of 14.9 ± 7.9, mean ± S.D., *n* = 11 with nine different donors), whereas a control 2000-kDa glycan polymer, LacNAc-PAA, did not bind (Fig. 4; data not shown). As was observed with the HEK 293 cell transfectants, binding of 2000-kDa 6'-sulfo-sLe^x-PAA polymer to human eosinophils was completely inhibited by polyclonal anti-Siglec-8 antibody (102 ± 20% inhibition, *n* = 11 with nine different donors), but not by any of the mouse monoclonal antibodies or the 30-kDa 6'-sulfo-sLe^x-PAA polymer (data not shown).

6'-Sulfo-sLe^x-PAA Polymer Uniquely Binds to Eosinophils in Whole Blood. Further evidence of the specificity of eosinophil binding to the 6'-sulfo-sLe^x glycan was provided by assays in which heparinized whole blood was incubated with biotinylated 2000-kDa 6'-sulfo-sLe^x-PAA or control polymer, and flow cytometry was performed after the addition of fluorochrome-conjugated streptavidin. As shown in Fig. 4, attachment of 6'-sulfo-sLe^x-PAA polymer to eosinophils was readily apparent, while no labeling of lymphocytes or neutrophils was observed and monocytes displayed a degree of reduced fluorescence. Interestingly, neutrophils and a

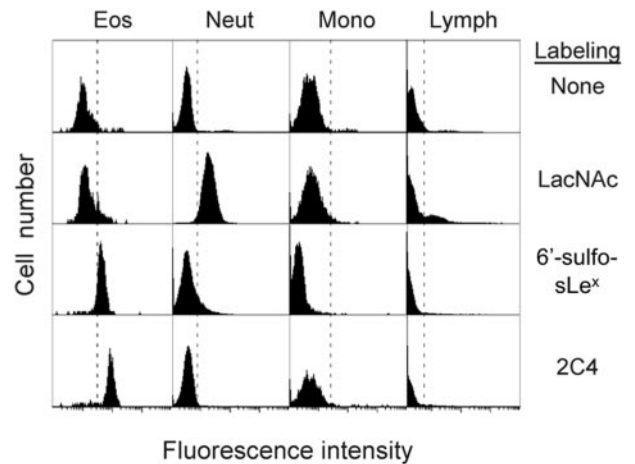


Fig. 4. The 2000-kDa 6'-sulfo-sLe^x PAA polymer selectively binds to eosinophils among leukocytes in whole blood. Attachment of the 2000-kDa 6'-sulfo-sLe^x PAA polymer and the 2C4 Siglec-8 monoclonal antibody to eosinophils was readily apparent, whereas no labeling of monocytes, lymphocytes, neutrophils, or basophils was observed. Binding of the LacNAc control PAA polymer is seen for neutrophils and a subset of lymphocytes. Results are representative of at least eight experiments with similar results.

subset of lymphocytes consistently and selectively bound the 2000-kDa control LacNAc polymer. As reported previously (Kikly et al., 2000), basophils were weakly labeled with Siglec-8 antibody, but no detectable labeling with 6'-sulfo-sLe^x-PAA polymer was seen (data not shown), presumably because of the low levels of basophil surface expression of Siglec-8.

6'-Sulfo-sLe^x-PAA Polymer Induces Eosinophil Apoptosis in Vitro. Engagement of Siglec-8 with monoclonal antibodies causes eosinophil apoptosis (Nutku et al., 2003, 2005; Nutku-Bilir et al., 2008). Therefore, it was of interest to determine whether binding of the 2000-kDa 6'-sulfo-sLe^x-PAA polymer had the ability to induce eosinophil apoptosis. As shown in Fig. 5, IL-5-primed eosinophils, which are known to be particularly sensitive to Siglec-8 antibody-me-

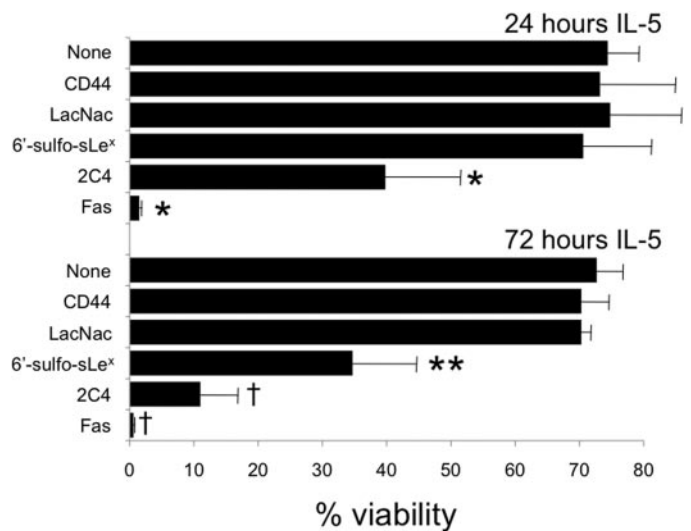


Fig. 5. IL-5-primed eosinophils undergo apoptosis when exposed to Siglec-8 antibody or 6'-sulfo-sLe^x-PAA polymer. Results represent means ± S.D. from five experiments at 24 h and three experiments at 72 h. *, *p* < 0.02 compared with the CD44 control; **, *p* < 0.03 compared with the LacNAc control; †, *p* < 0.005 compared with the CD44 control.

diated apoptosis (von Gunten et al., 2007; Nutku-Bilir et al., 2008), underwent apoptosis when incubated with 6'-sulfo-sLe^x-PAA polymer after 72 h of culture with IL-5 ($p < 0.03$), but not after 24 h of culture. The magnitude of the effect was less than that seen with the 2C4 anti-Siglec-8 monoclonal antibody and much less than that seen with the Fas antibody, both of which caused significant apoptosis after either 24 or 72 h of culture with IL-5. Enhanced sensitivity to polymer was not the result of altered surface expression of cell surface Siglec-8 during the culture because levels only varied slightly and in no particular pattern (data not shown).

Discussion

Although we and others have previously demonstrated eosinophil apoptosis with Siglec-8 antibody engagement (Nutku et al., 2003, 2005; von Gunten et al., 2007; Nutku-Bilir et al., 2008), our subsequent identification of a selective and specific ligand for Siglec-8, namely 6'-sulfo-sLe^x (Bochner et al., 2005), made it possible to test the hypothesis that a synthetic polymer decorated with this glycan would have the same cellular binding specificity and biology as that seen with monoclonal antibodies. With use of a soluble glycan-conjugated PAA polymer for initial proof-of-concept in the development of a nonbiologically based agent for detecting eosinophils and inducing their apoptosis, experiments revealed both selective and specific 6'-sulfo-sLe^x binding to Siglec-8 on transfected cells and on either purified eosinophils or eosinophils in whole blood. Affinity and avidity were sufficient to also mediate cellular adhesion to 6'-sulfo-sLe^x PAA polymer-coated surfaces. Glycan binding was inhibited by a sheep polyclonal antibody but not by any of our available mouse monoclonal antibodies. Although it was not as effective as the monoclonal antibodies, binding of 6'-sulfo-sLe^x-PAA polymer also induced eosinophil apoptosis. To our knowledge, this is the first time a soluble glycan has been used to selectively recognize and kill eosinophils and raises the possibility of developing enhanced glycan-based Siglec-8 ligands for diagnostic or therapeutic purposes in which eosinophil-specific targeting is desired.

The reason that 30-kDa 6'-sulfo-sLe^x-PAA polymer bound HEK 293 cell transfectants, but not eosinophils, remains unknown but is probably not due to cell surface sialic acid interference because their removal from the surface of eosinophils with sialidase had no effect. One possibility is that the larger polymer has the potential to span Siglec-8 over a wider area of the eosinophil surface or fortuitously expresses the glycan ligand in groupings that match the grouping of Siglec-8 on the eosinophil surface.

Although the 2000-kDa polymer does induce eosinophil apoptosis, it is not as effective as the antibody. The reason for this difference is unclear, but it may be the result of differences in multivalent versus polyvalent binding and the reduced flexibility of a dimeric antibody for inducing cross-links compared with a large, highly flexible PAA polymer. Indeed, the density of carbohydrate decoration is high, on the order of several hundred per PAA molecule. Further optimization of the polymer rigidity, size, and density of decoration with 6'-sulfo-sLe^x glycan may enhance both potency and efficacy for binding and induction of apoptosis. However, the goal of the present experiments was to achieve proof-of-concept regarding the ability of a soluble glycan ligand of Si-

glec-8, specifically a 6'-sulfo-sLe^x glycan, to selectively bind eosinophils among all other cells in peripheral blood, and to induce their apoptosis. This was important because subsequent to our publication showing specificity of binding of 6'-sulfo-sLe^x for Siglec-8 (Bochner et al., 2005), additional glycan array screens performed by the Consortium for Functional Glycomics with other lectins (www.functionalglycomics.org) suggested that more structures, including Siglec-7, might be capable of binding this glycan. The fact that no detectable binding to any other leukocyte subtype was seen, including monocytes, CD8⁺ T lymphocytes, and NK cells that express Siglec-7 (Nicoll et al., 1999; Angata and Varki, 2000), suggests that 1) endogenous binding sites may be masked by cell surface sialic acids, 2) binding specificity or affinity is less than that for Siglec-8, or 3) levels of Siglec-7 on these cells do not permit detectable levels of polymer attachment.

The closest functional mouse paralog to Siglec-8 is Siglec-F, which is also expressed on eosinophils and, like human eosinophils, preferentially binds 6'-sulfo-sLe^x (Tateno et al., 2005). Previous work showing that Siglec-F antibody administration to mice leads to profound and selective eosinophil apoptosis and depletion (Kearley et al., 2007; Zimmermann et al., 2008; Song et al., 2009) suggests that in vivo administration of the 6'-sulfo-sLe^x PAA polymer might have a similar effect, but unfortunately sufficient quantities are not yet available to perform such experiments. Other in vitro experiments with murine eosinophils have shown, however, that this same glycan-decorated polymer binds to murine eosinophils via Siglec-F and triggers its internalization via a pathway involving ADP ribosylation factor 6 but independent of dynamin and clathrin (Tateno et al., 2007). Additional experiments in vivo suggest the presence of a lung ligand for Siglec-F (Zhang et al., 2007). Its exact molecular identity remains incompletely defined but is currently under investigation (Bochner, 2009). Regardless, selective and specific engagement of Siglec-8 on eosinophils by natural or synthetic 6'-sulfo-sLe^x-containing ligands, glycomimetics, or antibodies could be useful in controlling eosinophilic inflammatory responses in vivo.

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